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STAIN TECHNOLOGY

JANUARY, 1931

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Editor and Business Manager

H. J. CONN

Soil Bacteriologist, N. Y. Agr. Exp. Station

Consulting Editors

CHEMISTRY: W. C. HOLMES Color Lab., U. S. Dept. of Agriculture

ZOOLOGY: S. I. KORNHAUSER School of Medicine, Univ. of Louisville

PATHOLOGY: F. B. MALLORY Boston City Hospital

BOTANY: W. R. TAYLOR Univ. of Michigan

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STAIN TECHNOLOGY

VOLUME VI JANUARY, 1931 NUMBER 1

PROGRESS IN THE STANDARDIZATION OF STAINS

STAINS FOR FROZEN SECTIONS

When staining frozen sections of fresh tissue one has not ordinarily been able to obtain double staining effects. Combinations of acid and basic dyes of the Leishmann or Giemsa type are not soluble in water, and frozen sections cannot be stained from alcoholic solutions. This fact, together with the difficulty in making permanent mounts from such sections, has detracted from the value of freezing technic.

For this reason special attention must be given to the paper by C. F. Geschickter and collaborators elsewhere in this issue,[1] in which a method of overcoming both of these obstacles is shown. They have accomplished four important things: they have found a method of dehydrating and clearing frozen sections without shrinkage or loss of color, and can thus mount them in balsam; they have shown how sections of fresh tissue can be pre-treated in buffer so as to secure staining pictures identical with those obtained on fixed tissue; they have found a solvent for stains by means of which acid and basic dyes can be mixed in any desired proportion; and thanks to this same solvent, can avoid the use of alcoholic solutions and thus apply a mixture of an acid and a basic dye to fresh tissue.

This method of dissolving stains may well prove important; and to prevent possible commercial exploitation of an undesirable sort, it is understood that a patent on it has been applied for. There is no intention, however, of allowing the patent to interfere in any way with the free use of the method in a scientific, non-commercial way.

The work reported by Dr. Geschickter and his co-workers is a development from the investigations of dyes in connection with cancer which are being made at Johns Hopkins Medical School under an appropriation from Mr. Francis P. Garvan, president of the Chemical Foundation. The work is being carried on in close coöperation with the Stain Commission; and it is planned that the stains and staining methods introduced shall be carefully standardized and made available to diagnosticians or other scientists interested in their use.

H. J. CONN

[1]Geschickter, C. F., Walker, E. P., Hjort, A. M. and Moulton, C. H. A new rapid method for tissue diagnosis. Stain Tech. **6**, 3, 1931.

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the October number of this journal.

Stains Certified August 15 to November 15, 1930*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Neutral red	NX 4	69%	As vital stain and in general histology	Aug. 25, 1930
Eosin Y	NE 7	89%	In general histology and as component of blood stains	Sept. 10, 1930
Eosin B	NEb 4	91%	As histological counterstain	Sept. 18, 1930
Light green SF yellowish	NL 5	75%	As histological counterstain	Sept. 22, 1930
Janus green	NJ 3	64%	As vital stain for blood	Oct. 23, 1930
Methyl green	NG 7	54%	In histology and as component of Pappenheim stain	Nov. 3, 1930
Martius yellow	NYm 2	79%	As histological counterstain	Nov. 10, 1930

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

A NEW RAPID METHOD FOR TISSUE DIAGNOSIS

CHARLES F. GESCHICKTER, EDWARD P. WALKER, *Surgical Pathological Laboratory, Johns Hopkins Hospital and University,* A. M. HJORT, AND CARL H. MOULTON, *Medical Research Department, Chemical Warfare Service, U. S. Army, Baltimore, Md.*

ABSTRACT.—**A new staining procedure applicable to both fresh and formalin fixed frozen sections is described in this report. The essential features are as follows:**

1. A buffer solution or prestaining bath, of the following composition, is used to collect the sections as they come from the microtome:

Potassium acid phosphate	**6.75 grams**
Normal sodium hydroxide	**30.00 cc.**
Distilled water	**570.00 cc.**
Glycerin	**200.00 cc.**
95% alcohol	**200.00 cc.**

The ingredients should be mixed in the order given.

2. The sections are then passed into the following staining solution[1] for 20 to 30 seconds: thionin eosinate 0.75 g., barium eosinate 0.25 g., azure A 0.25 g., dissolved in 100 cc. of a mixture of 4 parts ethylene glycol and 1 part ethyl alcohol (95%), plus 0.2% glacial acetic acid. Other stain combinations, using the same solvents, are under investigation.

3. The excess of stain is then washed off in two successive changes of 95% alcohol containing 20 cc. of glycerin to each 80 cc. of alcohol. The tissue is left in the first bath about 10 seconds and washed back and forth and is then transferred to the second bath for about 3 seconds. If the sections are too blue, wash longer in the second bath or add two or three drops of 5% glacial acetic acid to the first bath.

4. The section is next dehydrated by floating for 10 or 15 seconds in a solution of diethylene glycol monobutyl ether.

5. The section is now cleared in n-butyl phthalate by floating in this solution 20 seconds.

6. The section is now floated on a glass slide, blotted dry by means of photographic lintless blotting paper, and a cover slip upon which a drop or two of gum damar has been placed is mounted over it.

[1]This stain is known in this laboratory as No. 160.

Introduction

The present methods employed in staining fresh frozen sections are not entirely satisfactory. In most instances, these methods consist of an aqueous process of staining, using polychrome methylene blue (methylene azure) and mounting in glucose. Such methods do not give sufficient color differentiation in the various tissue components nor is permanency achieved. Other methods such as that advocated by Rhamy[2] employ alcohol, and hence, cause a marked and undesirable amount of shrinkage in the section.

A staining procedure adapted to frozen sections should have the following qualifications:

1. It should be applicable to both fresh and formalin fixed tissue.
2. It should be rapid.
3. Shrinkage or distortion in the section should be minimal.
4. Color differentiation should be marked.
5. The preparation should have clearness.
6. The preparation should be permanent if possible.
7. The technic should be simple and give uniform results.
8. The materials employed should be relatively cheap and obtainable in quantity.

An attempt to embody these qualifications in a more satisfactory method for staining fresh and formalin tissue by the frozen section technic is reported here. This attempt has yielded gratifying results in that improvement has been made at many points in the technic, even if perfection has not been attained. The principles involved and the details of the technic are presented below.

General Considerations

One of the most difficult achievements in the staining of fresh frozen tissue is that of securing a stain with sufficient color contrast. Aside from polychrome methylene blue (methylene azure) and several other thiazin dyes, no differential staining can be obtained from a single dye. It might be suggested that good differential staining is possible by dipping the sections in a series of dyes of different staining qualities. In general practice, however, this is not satisfactory because in some instances precipitation occurs, and in others, one dye decolorizes during the staining process with another. A polychrome effect may be secured by means of compound dyes; but with the exception of azure A plus Erie garnet B (Geschickter[3]), the majority

[2]Rhamy, B. W. Rhamy Triple Stain for Frozen Sections. J. Lab. and Clin. Med. 15, 490–492. 1930.

[3]Geschickter, C. F., Fresh Tissue Diagnosis in the Operating Room. Stain Techn., 5, 81. 1930.

of compound dyes are insoluble in water and in alcohol. If this obstacle of insolubility is to be overcome it is necessary to employ solvents other than alcohol or water which will serve to dissolve the compound dye without shrinkage or distortion of the tissue. A number of organic solvents and solvent mixtures which will serve this purpose are available, but have not been tried, to our knowledge, heretofore. Likewise, a number of compound dyes which have proven quite satisfactory for differential staining are easily prepared, and are available for use in frozen sections if such solvents are employed.

There are other factors quite as important as the stain and the solvents which must be considered in departing from traditional staining technics. With fresh frozen tissue, it is imperative to introduce measures which will minimize the effect of the time elapsing between the excision of the tissue and the staining of the cut sections. This time element is of importance because of the formation of lactic acid in excised tissue. The rate of lactic acid formation is more rapid in some tissues than others; hence, without corrective measures uniformity of staining would not be possible because of this resulting variation in the degree of acidity present. This time factor has been minimized in the technic herein advocated by the introduction of a KH_2PO_4–NaOH buffer solution of pH 7.0 as a step in the staining procedure. Another important factor involves the relative viscosities of the various solvents employed. A certain degree of viscidity seems essential to the proper handling of sections cut from unembedded tissues. Moreover in employing the groups of solvents necessary to a frozen section technic, transitional steps between the staining of the section and final mounting are important. If water is to be excluded from the mounted section, dehydration by some fluid miscible with water, but in which the dyes are relatively insoluble, and the use of a clearing reagent not miscible with the dye or water, but soluble in the mounting media are essential.

A certain amount of practice, of course, is always necessary in any technic before satisfactory results are obtained. In the procedures adopted here, floating the sections entirely free of the glass rod used for their conveyance, at each step, and avoiding folds in the section from the outset are important considerations.

The procedure advocated involves the following steps:

1. Securing the tissue block for the freezing microtome.
2. Freezing the block and cutting the sections.
3. Removing the cut sections from the microtome knife and collecting them in the proper solutions.

4. The staining procedure.
   a. Dipping the section in the prestaining bath or buffer solution.
   b. Dipping the section in the staining bath.
   c. Washing the section in the poststaining bath.
   d. Dipping the sections in the dehydrating bath.
   e. Dipping the sections in the clearing bath.
   f. Floating the sections on a glass slide.
   g. Blotting off the excess solvent adhering to section and slide.
   h. Mounting under a cover glass.

The steps enumerated above under 4, the staining procedure, comprise the novel features of the technic, but for the sake of completeness and for those relatively unfamiliar with the cutting of frozen sections, there is included below a short resumé of the sectioning procedure.

Cutting and Preparing Frozen Sections

The tissue blocks employed should be not larger than 3x10x10 mm. in size. Such blocks, with the broad face down, are placed in a few drops of water on the microtome stage and are then frozen by means of carbon dioxide gas. To conserve gas, the carbon dioxide should not be released in a continuous blast, but in small intermittent streams until the block is frozen. This conserves gas and gives more uniform freezing. Some tissues require only simple freezing, whereas others demand at least a thin wall of ice about them to permit smooth cutting. When frozen too hard, tissues have a tendency to crumble, hence, it is well to wait a few seconds after complete freezing before cutting the sections. The thickness of the cut sections should be between 10 and 20 microns, but for the beginner, perhaps, the latter size is to be preferred. The microtome knife should be very sharp if thin and unbroken sections are to be obtained. The Fanz automatic microtome knife sharpener in our hands, has proven highly satisfactory for keeping the knives in serviceable condition. At the end of each swing of the knife, the cut section is removed by sweeping a moistened finger tip downward, wiping the section from the blade, and carrying it on the finger into the water or some other appropriate solution to float the section. This solution is the collecting bath. The cut sections upon removal from the collecting bath are ready for the staining procedure. Successful staining and mounting is largely dependent upon having a smooth unfolded cut section at the beginning of the procedure.

Technic and Principles of the Staining Procedure

1. The Collecting Bath: It is customary to float the sections, as they come from the microtome into water, and to accumulate by rapid cutting a desirable number of such floating sections before beginning the staining procedure. The fluid used for floating the sections (the collecting bath) is usually either distilled or tap water, altho McCarthy of the Mayo Clinic advocates normal saline solution. In the present technic it has been found desirable to make the collecting bath serve a variety of purposes including partial dehydration, buffer action, and tissue preservation. For this reason the sections as

they come from the microtome are either placed in a special collecting bath, or if they are floated in tap water first, are immediately transferred to this special bath. Here they remain for varying periods of time, but if immediate staining is desired the sections are transferred after 2 to 3 seconds to the staining solution. A smooth unfolded section is secured near the tip of a glass rod about 5 mm. in diameter, after which the rod is tipped on a blotter or towel to remove the excess fluid, and then carried to the staining bath.

The prestaining bath is designed to have a buffer action. Tissues deprived of their circulation accumulate lactic acid. The formation of this acid is more rapid in some tissues than in others. The presence of lactic acid may modify the staining of tissue and thus contribute to nonuniformity. The introduction of a KH_2PO_4-NaOH buffer solution of pH 7.0[4] has been found to make the procedure uniform and also serves to intensify the staining, especially of the basic dye. Thus, sections may be stored several hours in this buffer solution with no appreciable change in staining quality.

The buffer solution is prepared by mixing 50 cc. of M/5 KH_2PO_4 with 29.54 cc. of M/5 NaOH and adding distilled water to a total volume of 200 cc. The KH_2PO_4 should be of the highest purity, and it is advisable to check the pH by some fairly accurate method. Such buffer mixtures varying in pH from 5.4 to 7.4 have been tried. The most suitable range is between pH 6.8 and 7.2 with 7.0 as, perhaps the optimum value. The pH 7.0 buffer has also been diluted 10 and 100 times but the diluted preparations are not as satisfactory as the undiluted. The pH 7.0 buffer has a total salt content of 0.37%.

While this buffer may be made up as indicated in a simple aqueous solution, it is preferable that the section before it is immersed in the stain should be partially dehydrated. This protects the stain from the precipitating influence of water and the addition of the dehydrating mixture also serves to act as a tissue preservative, in the advent that the staining is to be deferred, and the tissue held in the collecting bath for hours or days. The introduction of dehydrating agents into the collecting bath may be accomplished by various methods. Alcohol alone will cause shrinkage, but mixtures of alcohol with glycerin, ethylene glycol, or diethylene glycol serve very well.

If simplicity is desired, one part each of alcohol and glycerin (using 95% alcohol) may be added to 8 parts of the aqueous buffer solution described above. This combination of alcohol, glycerin and buffer solution has worked very well in our hands, but there is a slow but progressive introduction of water into the staining bath if such a collecting fluid is constantly used. Furthermore, if this buffered solution is used as a collecting bath for the sections as they come directly from the microtome, care must be observed in rinsing the finger tip before carrying it back to the microtome knife, because the buffer corrodes the knife edge. Thus to avoid injury to the knife and to the stain the collecting bath may be divided into three steps instead of one. The sections may be floated in the tap water, transferred to an aqueous buffer solution, and thence to a solution containing a mixture of equal parts of glycerin and alcohol, rather than employing a single collecting bath combining aqueous buffer, alcohol and glycerin.

2. The Staining Bath: From the collecting bath the section is floated off from the rod into a dish of stain, and after 20 to 40 seconds

[4]Clark, W. M. The Determination of Hydrogen Ions 3rd Ed. Williams and Wilkins, Baltimore. See p. 200.

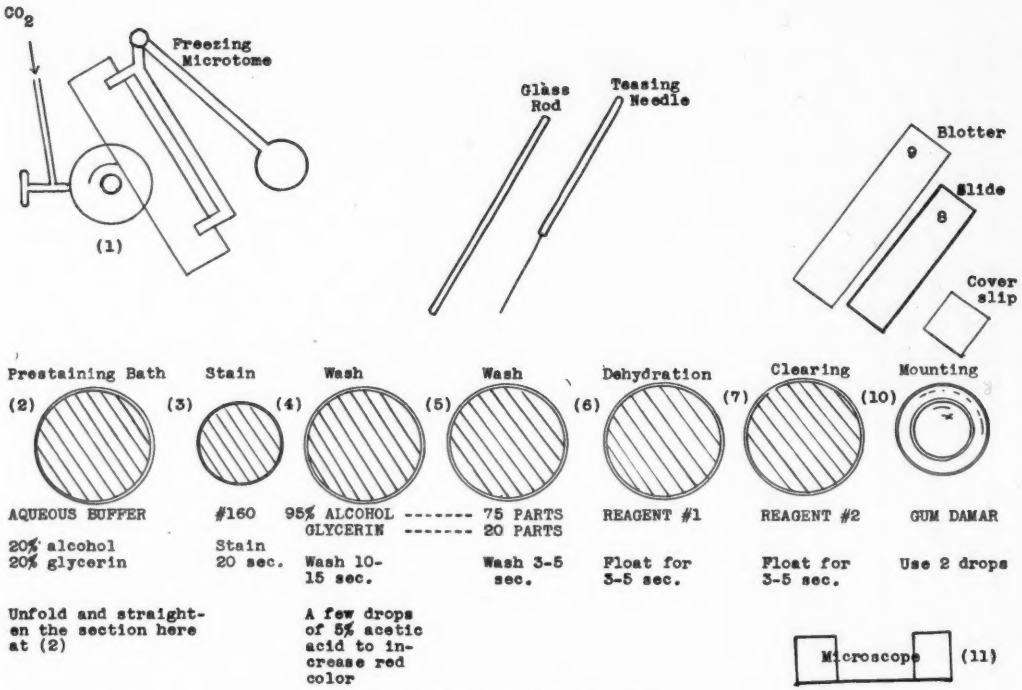

Fig. 1. Diagram of staining technic.

Above timing is that originally employed by the writers: it is now preferred to dehydrate (step 6) 15 seconds and clear (7) 20 seconds.

of staining is again lifted with the rod and it is then ready to be transferred to the solutions used for washing and decolorizing.

The staining bath, in addition to its dye content which is designed to give contrasting color effects, must be free from precipitate, and must not shrink the tissue. This is dependent upon both the type of solvents and the kind of dyes selected. In certain compound dyes of which eosin is a component, differential stains have been obtained which compare favorably with the hematoxylin-eosin preparations employed in staining fixed sections. These eosin compounds are insoluble in water but are easily carried into solution by a group of organic solvents.

Some of the varieties of staining mixtures employed with success are the following:

A. Thionin eosinate, 1% in any of the following solvent mixtures:
Glycerin 50 parts; ethyl alcohol (95%) 50 parts
Diethylene glycol 70 parts; ethyl alcohol (95%) 30 parts
Ethylene glycol 80 parts; ethyl alcohol (95%) 20 parts

B. Thionin eosinate 1.0 g., and barium eosinate 0.5 g., to each 100 cc. of any of the following solvent mixtures:
Glycerin 50 parts; ethyl alcohol (95%) 50 parts
Diethylene glycol 70 parts; ethyl alcohol (95%) 30 parts
Ethylene glycol 80 parts; ethyl alcohol (95%) 20 parts

C. Thionin eosinate 0.75 g., barium eosinate 0.25 g., and azur A 0.25 g., to each 100 cc. of any of the following solvent mixtures:
Glycerin 50 parts; ethyl alcohol (95%) 50 parts
Diethylene glycol 70 parts; ethyl alcohol (95%) 30 parts
Ethylene glycol 80 parts; ethyl alcohol (95%) 20 parts

The barium eosinate is first dissolved by the aid of heat, and then with continued heating the thionin eosinate is added and after about 5 minutes with the temperature held at slow boiling (care should be observed here to avoid ignition of alcohol vapor) and with continuous stirring, the hot preparation is filtered. The filtrate is employed, when cool, in staining. The filter paper should contain practically no solid matter. Eosin may be substituted for the barium eosinate but it is not quite as soluble in these solvents as the latter, also azur A may be added to the cooled solution. There is not much to choose between the solvents here employed, but the ethylene glycol mixture has been used most often by us.

In many instances thionin eosinate alone gives perfectly adequate staining, but often the red element is deficient. In such cases (B) and (C) are to be recommended. However, the red element may be accentuated without adding eosin or barium eosinate by simply adding a drop or two of glacial acetic acid to each 100 cc. of the stain. If the dye is to be used routinely for both fresh and formalin fixed tissues, the addition of the glacial acetic acid is essential. Stain C with glacial acetic acid added (0.2 cc. per 100 cc.) is applicable to all tissues including paraffin and celloidin sections.

3. Post-Staining Baths for Washing: It is customary to follow the staining of tissue by washing the excess in water. In addition, it is

customary to employ some solution as a differential agent to bring out the most desirable elements in the stain.

These can both be accomplished by a single bath and in addition, in the interest of permanency, this bath can contain solutions which will replace the aqueous content of the tissue. The bath used for washing the tissue is best poured into two separate dishes, altho one will suffice. The section, as it comes from the stain, is carried on the glass rod and floated into the first dish and washed back and forth for 10 seconds. It is then transferred by means of the glass rod to the second dish where it is allowed to float until the desired amount of red color becomes evident.

Since in the staining technic herein advocated, the stain contains no water, the dehydrating effects of the staining solution are lost if we revert to an aqueous post-staining bath. Water may, however, be used to wash off the excess dye before passing into the post-staining baths described here, if in the interest of economy it is desired to keep the latter relatively free from dye. The solvents employed as this bath, are designed to dehydrate the tissue further, with minimal shrinkage and without injurious effect to the cells. This post-staining bath is also miscible with the stain and with the dehydrating bath and final clearing reagent. A solution containing 20 parts of glycerin and 80 parts of 95% alcohol is used. This bath rids the tissue of excess dye and its viscosity prevents shrinkage. The mixture is also relatively inexpensive, readily made up, and can be discarded when dirty. Diethylene glycol may be substituted for glycerin in these baths, using 25 parts to 75 parts of 95% alcohol. We prefer this reagent to glycerin. A few drops of 5% acetic acid may be added to the first dish if a stronger red color in the sections is desired.

4. The Dehydrating Bath: After being removed from the post-staining bath the section is floated into the dehydrating reagent. This dehydrating bath must come up to certain definite standards. It must be miscible with water and with the final clearing reagent. It must have sufficient viscosity to prevent shrinkage of tissue and it must also be a relatively poor solvent for the stain used to prevent decolorization of the section. Many new organic solvents have been tried in an endeavor to meet these requirements. The best possible answer has probably not been found at the present writing, but several new organic solvents serve the purpose very well. Diethylene glycol monobutyl ether is the compound used in the present technic. Ethylene glycol monobutyl ether and ethylene glycol monoethyl ether have also been tried with less satisfactory results. Diethylene glycol monobutyl ether has sufficient viscosity, is miscible both with

water and with the clearing reagent used, and is relatively cheap. It does not decolorize the section and its reaction is not injurious to the stain. The sections are floated into this solvent, just long enough to free them from the rod (10 or 15 seconds) and are then carried to the clearing reagent.

5. *The Clearing Bath:* The cut section is freed in this bath where it is permitted to remain for 15 to 20 seconds after which it is floated onto a glass slide.

This bath serves the purpose of removing the other solvents employed and to remove any remaining traces of water. It must not dissolve the stain, shrink the tissue, nor interfere with mounting; hence it must be miscible with the dehydrating and the mounting agent, and must also possess a certain degree of viscosity. The cut sections released in this bath should spin out flat on the surface. At present, n-butyl phthalate has proved the most satisfactory for this bath.

6. *Mounting:* After the cut section is floated smoothly onto the glass slide, the excess fluid is carefully blotted off by means of photographic lintless blotting paper, or bibulous paper. A drop or two of gum damar, neutral Canada balsam, or neutral cedar oil is placed on a cover slip which is then inverted and applied to the blotted section on the slide; gum damar is preferred on account of its neutrality. The preparation is now ready for examination under the microscope.

Materials and Apparatus

The eosinate dyes used in this technic have been prepared especially for the Surgical Pathological Laboratory of the Johns Hopkins Hospital and University by W. C. Holmes of the Color Laboratory of the United States Department of Agriculture. The dyes will be handled commercially by the Abbott Laboratories, North Chicago, Illinois, who will also act as agents for distributing all other materials and reagents described in this technic, with the exception of the following:

The Fanz automatic microtome knife sharpener, procurable from Arthur H. Thomas Company, West Washington Square, Philadelphia, Penna.

Stender dishes (five 60 mm. x 35 mm. dishes are used for reagents and one 50 mm. x 30 mm. is used for the stain) procurable from any biological supply house.

Bibulous paper or filter paper (fine grade used for blotting) procurable from any biological supply house.

Frozen Section Microtomes—any one of the following standard makes:

The Spencer Automatic No. 880
The Sartorius
The Leitz or
The Bausch & Lomb.

The Eastman Kodak Research Laboratories of Rochester, New York have supplied us with the organic solvents used in developing this technic and have generously coöperated in helping us solve the problem.

General Applications of the Technic

In the abstract preceding this paper and in the diagrams shown, the technic as used routinely in the Surgical Pathological Laboratory of the Johns Hopkins Hospital and University is given. In this laboratory, the technic as outlined is applied both to fresh, unfixed tissue and to formalin fixed material. The same staining procedure is also used on celloidin sections, except that it is found advantageous to add a little more glacial acetic acid to the staining solution. The procedure is also adaptable to paraffin sections. The sections are deparaffinated and put thru Lugol's iodine solution and alcohol as for ordinary staining if bichloride of mercury has been used in fixing. Then the dye is applied with a dropper and the section stained for 15 to 30 seconds. It is then run rapidly thru the washing, dehydrating, and clearing reagents as outlined for frozen sections. It is important, however, after deparaffinating the sections to rinse them before staining in distilled water.

THE DISSECTION STAINING AND MOUNTING OF STYLES IN THE STUDY OF POLLEN-TUBE DISTRIBUTION

JOHN T. BUCHHOLZ, *Univ. of Illinois, Urbana, Ill.*

ABSTRACT.—**The methods here outlined have been given in general in previous publications; but they are now summarized, with special reference to *Datura* styles, for the benefit of those desiring further details.**

Pistils, artificially pollinated and kept at 18 to 22° C, are slit along two sides, scalded 30 seconds to 2 minutes in water at 70 to 75° C, and killed by immersing for several hours in 50% alcohol containing 6% formalin. Within 12 hours of killing, they are dissected with needles and forceps under a wide field binocular microscope, removing the cortex from style and stigma. The strand of conducting tissue thus separated from the cortex is stained within 24 hours of dissection in a watch glass containing a mixture of 8 parts 1% aqu. acid fuchsin and 2 parts of 1% aqu. or alc. light green. Staining requires 3 to 6 hours, and may be continued over night if stain is not too concentrated. The strand of tissue is cleared several hours or over night in 80% lactic acid, carefully spread out on a slide, and mounted in lactic acid. After a few days the preparations are sealed around the edges of the cover glasses with damar in xylol or paraffin to which gum mastic has been added.

Directions are given for counting the ends of pollen tubes and for keeping the records when genetic studies are being made.

In a technic for the study of pollen-tube growth, it is desirable to have, mounted on a single microscope slide, the entire strand of tissue thru which the pollen tubes grow. If this can be done, the study may become quantitative. Methods of dissection may achieve this end, which is defeated more or less by serial sections cut in paraffin. The original discovery of this technic was made in an effort to circumvent the paraffin method.

The method has become very useful for the study of pollen-tube growth in *Datura* where the conducting tissue of the style is well developed and easily separated from the cortical tissue. I have tried it out casually on *Oenothera* with some success and also on *Nicotiana* and a few other members of the Solanaceae. It is highly successful with *Cobaea scandens*, and I believe, for the styles of any plant among

dicotyledons in which there are many ovules per ovary, a condition which is usually associated with a large strand of conducting tissue.

I am equally convinced that with plants having single ovules or only a few ovules per ovary, the method will not be found successful, as for instance with maize or with grasses generally, where a special technic of a different kind must be worked out. Liliaceous plants which often have hollow styles whose cavities are lined with the stigmatic cells have not been investigated, but these would seem to offer difficulties.

Several other investigators have worked out special methods. For example, Miss Sawyer[1] has found it possible to separate strands of pollen tubes from the styles in *Iris*, but whether her method is adapted to quantitative studies is not clear. East and Park[2] have used the paraffin method in a study of pollen-tube distribution in *Nicotiana*. The method described here is a special one useful in the study of pollen-tube growth in plants having the structure described. Special methods may afford valuable hints which may be adapted to similar problems in other plants, and it is hoped that the details of my technic and experience may be found either directly or indirectly useful to other investigators.

While the general outline of the technic used in the study of pollen-tube growth has been included in several publications[3], frequent requests have been made by various individuals concerning further details. In view of the fact that certain improvements have been made since these publications, I have decided to prepare a more complete outline of this technic, and in this account I am describing it with special reference to *Datura*.

Pollination Methods

When flowers of *Datura* are used for the study of pollen-tube growth, it is essential to have the stigmas fully receptive and pollinated evenly. In natural self-pollinations and when the pollinations of routine crosses are made, the pollen is usually applied in large masses

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[1]Sawyer, M. Louise. Crossing *Iris pseudacorus* and *I. versicolor*. Bot. Gaz., **79**, 60–72. 1925.

[2]East, E. M., and Park, J. B. Studies on self-sterility II, Pollen-tube growth. Genetics, **3**, 353–366. 1918.

[3]Buchholz, J. T., and Blakeslee, A. F. Studies of the pollen-tubes and abortive ovules of the Globe mutant of Datura. Science, **55**, 597–599. 1922.

Pollen-tube growth at various temperatures. Am. J. Bot., **14**, 358-369. 1927.

Pollen-tube behavior with reference to sterility in Datura. Mem. Hort. Soc. of N. Y. **3**, 245-260. 1927.

and sometimes before the style is fully receptive. Massing of the pollen should be avoided if quantitative tests of pollen-tube growth are to be attempted. The pollen should be taken fresh from the anthers about to shed or from a flower in which the anthers have recently opened, and applied with a No. 1 or No. 2 sable brush to fully receptive stigmas of previously castrated flowers. The use of a brush insures even distribution of the pollen, and experience will soon demonstrate the amount of pollen which should be used. If too much pollen is used, part of it may remain ungerminated due to crowding. In pollen-tube growth tests on *Datura*, we usually apply between 600, and 800 or 900 pollen grains with a No. 2 or No. 3 sable brush and consider it a fairly abundant pollination, and with a No. 1 or No. 2 sable brush less than 400 which may be considered a moderately sparse pollination, depending somewhat upon the size of the individual stigma which is used. It is obvious that the pollen grains must be brought to germination very promptly and as nearly simultaneously as possible if a test of their distribution is expected to show any significant differences in the growth rate of pollen tubes. If the pollen grains in a normal plant germinate evenly, a distribution curve similar to Fig. 10 may be obtained from a test of 8 to 16 hours, the length of the diagram depending more or less upon the temperature.

Humidity is an important factor in pollen germination. A glass bell placed over a set of flowers or a closed straw tied over the stigma, giving an increase in the relative humidity will usually result in good pollen germination. These conditions may give germination as nearly simultaneous as possible, but a saturated atmosphere around the dew point is not desirable. Low temperatures around 18° C are better for germination than high temperatures, and constant temperature conditions are very desirable in controlling the rate of pollen-tube growth. For these reasons, it is desirable to use cut flowers, brought in just before the pollinations.

Structure of the Style in Datura

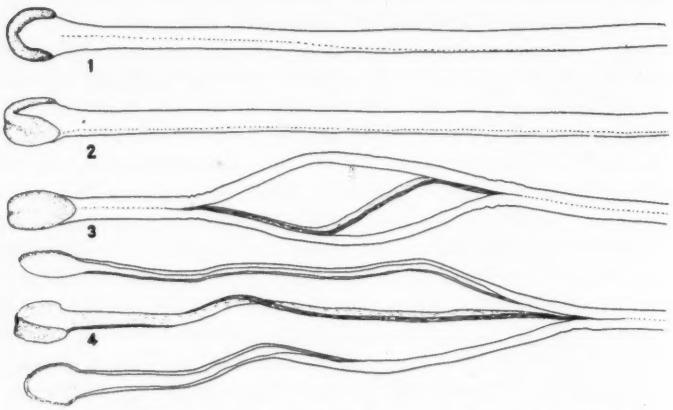
Figs. 1 to 4 show the stigma and style of *Datura*. It will be noted that the core of conducting tissue which is removed by dissection is continuous with the stigma, and is surrounded on all sides by cortical tissue which must be completely removed if the pollen tubes are to be observed. There are several preliminary operations which facilitate its removal.

Slitting and Killing the Styles

After a suitable interval following pollination (16 hours at 18° C or 10 hours at 22° C for *Datura*) the styles should be broken off above

the ovary and killed. A great deal of time and trouble is saved in dissection if the cortex of the style is first slit along two sides as shown by the dotted line in Fig. 2. Dissection is further facilitated by scalding. The process of scalding serves several purposes. It kills the pollen tubes and thereby arrests their growth or contraction, and it renders the previously turgid cortex tough and pliable. The scalding also renders the conducting tissue soft and causes its cells to remain better attached to each other. The slitting facilitates a more rapid penetration of the killing fluids.

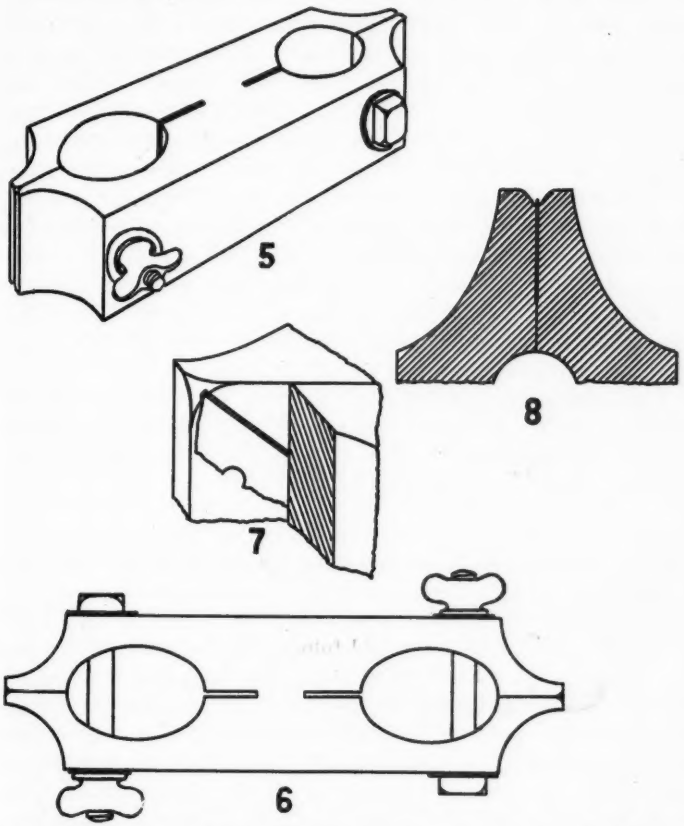
The slitting may be done by dragging the style thru a groove in a block of wood in which the fragment of a razor blade is embedded. I



Figs. 1–4. Stages of the dissection of styles of Datura. The dotted line in figure 2 shows the correct position for the slit cut by the aid of the apparatus shown in figure 5. Figure 1 has this slit in the wrong position. The central strand of conducting tissue, which is removed by dissection, is shown in the two lower figures and merges into the stigma.

have recently designed an apparatus for this purpose which may be easily adjusted, shown in Figs. 5 to 8. This consists of a well-seasoned block of hard fine grained wood (maple preferred) cut from a 1¼ inch board with a scroll saw into the shape shown in Fig. 6. The screws permit adjustment or removal of the cutting knife, which is located at the bottom of a very small groove, filed between the corners where the two halves come together. It should not cut deep enough to reach the conducting tissue. From the middle of the bottom of the groove a piece of a razor blade introduced in the space between (Fig. 7, 8) is allowed to project just far enough to cut about half way thru the cortex. This must be adjusted by trial. Very gentle pressure of

the fingers holds the style in place, a slight rolling action tending to adjust the style if it begins to twist and turn in such a way that the groove would come on the wrong side, as shown by the dotted line in Fig. 1. Each style should be slit on the sides as shown in Fig. 2 and as near to the stigma as convenient but the last 2 or 3 mm. of this cut should be made with a needle during the process of dissection. It might be completed on the slitting block but the stigma may thus be



Figs. 5–8. Fig. 5 is the style-slitting apparatus. This is approximately six inches long, cut from block of 1½ inch maple, following the proportions shown in the pattern of Fig. 6. Fig. 7 shows the position of the piece of razor blade used as a cutting knife. The corner is allowed to project into the groove far enough to cut partly thru the cortex of the style. Fig. 8 shows the groove and cutting knife in an oblique section passing thru the groove.

injured. I prefer to leave the stigma untouched as much as possible, in order to prevent the loss of any ungerminated pollen grains. The entire process of slitting a style with two grooves on opposite sides as shown in Fig. 2 need not require more than 10 to 20 seconds, and the style thus treated is dropped immediately into scalding water.

The scalding may not require more than 30 seconds; at times we have left them for as much as 2 minutes. The temperature of the water should be between 70° and 75° C, and the styles are removed as soon as they begin to darken and placed in a killing solution of formalin-alcohol (formalin 6%, alcohol 50%) in a watch glass, where they are left, with their labels, for one or several hours until dissection.

At times the styles are easy to dissect with a wide range of differences in treatment and at other times they are difficult to dissect and many variations of treatment have been tried without success. These are details which seem to depend upon the condition of the material. If the styles for pollination are selected from plants at the time when they are setting seed capsules freely, the dissections are more easily made than from collections earlier in the season when seed capsules are not setting. For practice in the art of dissection the styles taken from wilted flowers of open field pollinations, about the second day after their first opening, are best, but the slides from these can have no value, since most of the pollen tubes have completed their growth long before this. They are recommended only as specially favorable material which affords the beginner a means of becoming familiar with the process of dissection itself.

Dissection

For dissection a wide-field binocular dissection microscope is desirable. The older models of Greenough binoculars have a small field. Several manufacturers are now supplying wide-field binoculars which enable the operator to see a field fully three times the diameter of the older models. A revolving nosepiece which permits a quick change from low to higher power with par-focal sets of lenses is desirable. I prefer to work with the lower powers (about 10 × or 12 × magnification and turn to a high power (20 × or 30 ×) only when necessary.

While I have preferred to work with combination of transmitted and reflected light from a desk lamp providing oblique illumination and a mirror or white field giving subdued transmitted light, some of my assistants have found the illumination of a desk lamp alone more desirable. One of them preferred direct sunlight with the object over a black background.

The dissection instruments should be selected with care. Fine straight-pointed forceps should be selected, the springs ground thinner and the points finer. If the springs are too stiff the light pressure necessary in holding the object without injury cannot be regulated. The dissecting needles should be made by inserting No. 10 or No. 12 sewing needles in holders and bending the last 2 mm. slightly by heating them in a flame. This heating and bending must be done quickly in order to prevent oxidation as much as possible. As soon as the point is red-hot, it should be pressed quickly against a firm surface. It is well to polish the oxidized surface in some way, for instance by pushing the point in and out of a piece of closely-woven woollen clothing, or by rubbing it against a piece of pine.

The dissecting should be carried out within 12 hours after killing. A day later it may be very difficult if not impossible, and at times I have found it advantageous to work on the material as soon as the killing agent has had time to penetrate into the tissues.

In the detailed procedure, the following steps should be taken. Place the styles from the killing fluid into water in the cover of a Petri dish placed on the stage of the microscope. Hold the style with the edge of the stigma toward the observer (Fig. 2), and with the needle, complete the slits represented by the dotted line up to the edge of the stigma (care should be taken thruout not to rub ungerminated pollen grains from the stigma). Then insert the needle and points of the forceps into the slit at a point 10 to 15 mm. from the upper end, and as the opening is made and lengthened, carefully separate all of the conducting tissue from the inside of the cortex on one side. Fig. 3 shows it partially separated from both sides in different places. Grasp the conducting tissue and cortex on one side with the forceps and gradually widen this gap by hooking the curved point of the needle into the cortex of the other side, working the opening upward toward the stigma. As the separation approaches the stigma the cortex which is being loosened should be bent sharply upward and outward. Skilful manipulation at this point, taking a fresh hold when necessary, will remove the lateral lobe of the cortex at the stigma very easily, and very neatly, except for an unimportant marginal fringe of the stigmatic cells. Fig. 4 shows a stigma and the conducting tissue with the cortex removed on both sides. The strip of cortex so loosened may now be stripped off from the lower portion and discarded. If much less than half of the circumference of the cortex was removed, another narrow strip should be separated and pulled off before the final removal of the conducting tissue from the remaining part, especially if it is found that the entire lobe of the cortex was not removed from one side at the stigma.

Different individuals carry out these dissections successfully with slight variations in the procedure. The important fact to be kept in mind is that the entire strand of conducting tissue should be removed in one piece. In the final removal the conducting tissue should be grasped very lightly with the forceps and the cortex hooked with the needle, not the reverse. When the dissection is complete, the strand of conducting tissue is lifted on the needle and placed back in the watchglass with its label.

Staining

Staining is done in a watchglass a few hours or not less than a day after dissection. As given formerly, I used "magenta"[4] from an old stock of stains, with a little light green added. When the supply of this was approaching exhaustion Miss Lillian Martin, my technical assistant at that time, made a series of tests of various stains—acid fuchsin, basic fuchsin, light green and several other stains such as eosin yellow, Janus green, anilin blue, Berlin blue, alone and in combination or as mixtures. The result showed that basic fuchsin, which is also called magenta, is not as satisfactory as acid fuchsin with light green added. The stain mixture which results is not exactly the same as that of the magenta mentioned in our early publications but it is practically as good. More recently I have tested fast green added to the acid fuchsin, and found it much less satisfactory. To 80 cc. of a 1% aqueous solution of acid fuchsin, add about 20 cc. of a 1% solution (alcoholic or aqueous) of light green S. F. Yellowish[5]. The resulting color will approach a violet shade. The effect of the light green seems to be two-fold. Pollen tubes are stained very much darker than in acid fuchsin alone, and when the best combination has been found the empty exines of the germinated pollen grains gradually take on a pale blue color after the slide has cleared several days. Staining may be completed in 3 to 6 hours. Over-night staining (10 hrs.) is satisfactory if the stain is not too concentrated. Thruout the process of staining and clearing, the labels for each specimen are kept in an empty watch glass, which serves as a cover for the watchglass with the specimen.

Clearing and Mounting

The conducting tissue is lifted from the stain with a needle, allowed to touch a clean piece of absorbent toweling or filter paper to remove the excess of stain, and placed directly in a dish of 80% lactic acid for clearing. After several hours or a longer indefinite

[4]Purchased from Eimer and Amend in 1921. It proves to be neither acid nor basic fuchsin.

[5]These were certified stains bearing the certification numbers N R 4 and N L 4 respectfully.

period (over night) the strand of tissue is picked up with a fine brush by its stigma end, using a rolling motion, and spread out on a slide. It should be spread out straight and flat. Care should be taken to avoid twists which may usually be removed by passing the brush downward along the sides of the strand. More lactic acid is added and a cover glass applied. Gentle pressure will spread out the tissue into a thin layer, about 2 or 3 mm. wide, the stigma being spread much wider, in proportion to its size.

Considerable displacement of pollen tubes occurs in spreading but this is chiefly lateral. The ends of the pollen tubes will occupy very nearly their original distance from the stigma and ovary, and since their linear distance of growth is the point of chief interest as in the study of distributions (Fig. 10) there is very little distortion from this source.

The pollen tubes are visible after mounting and flattening, but will be much more conspicuous after a few days, when clearing has been completed. Overstained slides may sometimes be improved by heating a few hours in an oven at 60° to 70° C, but this must be done before they are sealed.

Sealing

After a few days the slides may be sealed along the edges of the cover glass. Damar in xylol applied with a sable brush is a fairly satisfactory seal. Paraffin with considerable gum mastic added is also a good wax for sealing, one which cools quickly and may be applied easily with a heated wire dipped into the wax. A wire 2 mm. thick with a loop for a handle and the other end bent at right angles into an L, may be heated and its horizontal end touched into the wax, then applied parallel to the edge of the cover glass, thus sealing an inch or an entire edge of the cover in a single operation.

Cover glasses No. 2, 12 to 18 mm. wide and of suitable length are desirable since the wide margin left on the slides offers a substantial surface for sealing. For styles of extreme length the 35 × 60 mm. cover glasses may be cut and used. A chip from a carborundum hone fastened with a hard sealing wax (De Khotinsky's cement) to the end of a handle serves admirably as a glass cutter for these cover glasses and is preferable to a diamond, because the pressure is not so concentrated at a single point, which may cause shattering. Cutting cover glasses without considerable loss thru breakage requires considerable technical skill, and should be done on a perfectly smooth hard surface, such as a piece of plate glass or fine carborundum hone. Of course supply houses can furnish the desired sizes, cut to order.

Counting Pollen Tubes

If studies of pollen tubes are made for genetic work, it may be desirable to count and to plot the distances reached by the pollen tubes. The forward ends only should be counted and a little careful observation will enable one to distinguish broken ends of tubes from the actual ends which are rounded. I use a mechanical stage with millimeter scale and count the pollen tubes visible in the field between two parallel cross hairs in the ocular, so spaced that 2/3 mm. on the slide is included when the 8 mm. apochromatic objective is used with a No. 2 ocular. The use of a 4 mm. achromatic objective will give one about the same detail, for a field half as wide. A sheet of 9 x 11 inch paper having square rulings 5 mm. apart may be used for the record. The transectional counts for scattered pollen grains may be made with a low-power lens down to a level of a selected point of reference at the top of the stigma. Then the high-power lens is used and the pollen grains and pollen tubes which are visible in the transects are counted differentially. They are entered on the record (Fig. 9) in four columns, showing the two kinds of pollen grains observed, and two classes of pollen tubes. A pair of hand tally counters are used to facilitate the counting. In the stigma region it is a safer method to pass twice over each transect; the first time, counting the two classes of ungerminated pollen grains, and the second time, counting the normal and the abnormal pollen tubes. Only the ends visible in the field between the cross hairs are counted in adjacent transverse sections. The record of the counts gives four vertical columns of figures for the stigma region. Two columns continue beyond the stigma region down the style as long as any ends of pollen tubes are visible. At the point of beginning the count on the stigma, the reading on the mechanical stage is recorded, and again every 4 or 6 mm. This makes it possible to make recounts in certain portions of the style without going back to the beginning. For example, in Fig, 9 there were two burst pollen tubes and one normal pollen tube when the scale reading was at 16.3 (see middle column, near top). It is possible, therefore, to insert the slide on the same mechanical stage at this reading and recount the spaces beginning at any such point and continue the checking as far beyond as desired.

In plotting the distributions, the two columns of ungerminated pollen grains are added and all plotted together as a vertical bar at the extreme left. There may sometimes be a significance in finding burst pollen grains on the stigma rather than rounded ungerminated pollen grains. If so, the original record will contain this information

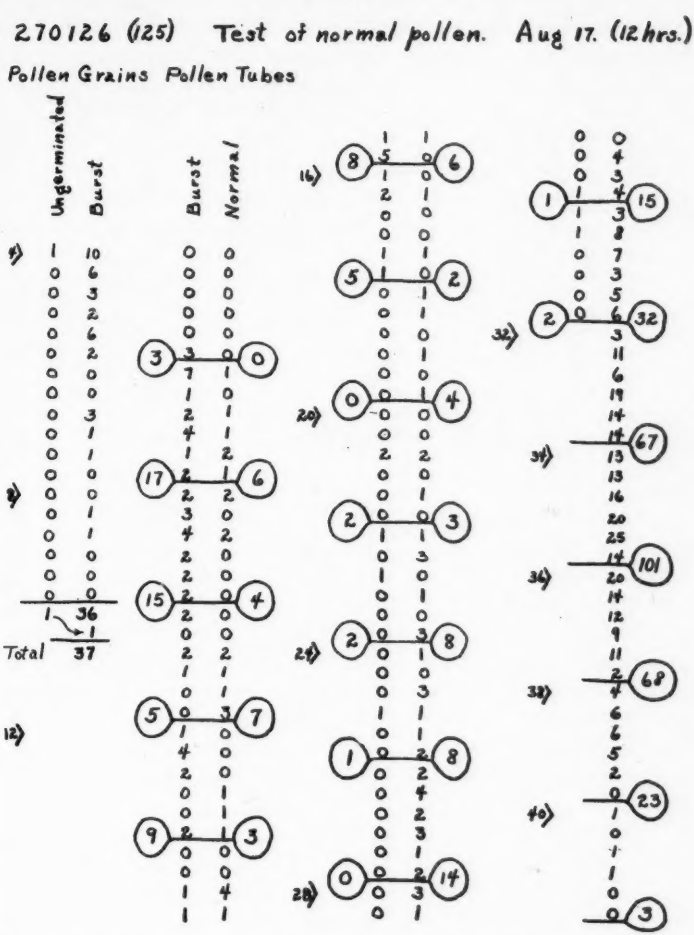


Fig. 9. Data sheet showing method of recording in columns the number and condition of pollen grains and ends of pollen tubes as they occur in successive 1/3 mm. transects beginning at the stigma. The figures within the loops represent the number of tubes in each 2 mm. space and are plotted in the diagram (Fig. 10). Readings of the vernier of the mechanical stage are shown at intervals alongside the columns. Reduced from a 9 x 11 inch record sheet.

if this plan of record is followed, but we have usually plotted both of these groups together.

The intervals showing pollen-tube distributions are then added, as shown by the figures within the loops on the record sheet of Fig. 9.

If an 8 mm. lens was used with the cross-hairs of the ocular 2/3 mm. apart these divisions are made between every third space; if 4 mm. objective was used and the spaces each represent 1/3 mm., six of them are added together, letting each space which was plotted represent a 2 mm. length of the style. All pollen tubes which were recorded as abnormal in appearance (left column) are plotted below the datum line, and normal appearing tubes above this line. In some of our earlier publications we have used 3 mm. spaces, but have since found it better to use the 2 mm. spaces, since the vertical scale of the diagram is more satisfactory. For *Datura*, it is not desirable to plot spaces less than 2 mm. The stigma is a curved surface, sometimes

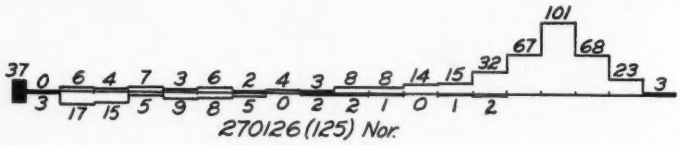


Fig. 10. Completed diagram of pollen-tube distribution in a test of the pollen of the normal plant used in Fig. 9.

longer than wide, and the pollen grains themselves are not at the same distance from the ovary. This curvature gives some distortion. For example, even if 100 pollen tubes had grown out to exactly the same length after a period of 12 hours, their points of origin on this curved surface would vary considerably, and their forward ends would be at different distances from the stigma. However they would nearly all be observed and recorded within two adjacent 2 mm. intervals, which is close enough to show most bimodal groupings if any are found. This difficulty may be overcome in part by selecting styles with short stigmas, and also by pollinating only the upper part of the stigmas, but we have done this only in certain special tests.

A METHOD FOR STAINING POLLEN-TUBES WITHIN THE PISTIL

CLYDE CHANDLER, *New York Botanical Garden, N. Y. City.*

After testing various methods and numerous stains for securing differential staining of pollen-tubes within the pistils of plants the following method has been found highly satisfactory. Pistils which are to be studied in relation to pollen-tube growth are collected from the plant at intervals after pollination as desired and placed immediately in a killing solution made by adding 6 to 7 cc. of commercial formalin (37%) to 100 cc. of 70% alcohol. Pistils placed in this solution may be examined soon after their collection or they may be stored for future study.

For pistils with a central canal (*Hemerocallis* and *Lilium* for example) the method of dissection and staining is as follows: First the entire style is placed on a glass slide and by means of two very fine needles the style is split longitudinally along one side to the central canal. This is most easily done by beginning at the stigmatic end and cutting downward. The cut surfaces are spread apart until they lie flat on the slide. This dissection, if carefully performed, leaves the group of pollen-tubes in place. A drop of aceto-carmin (saturated solution of carmin[1] in warm 45% acetic acid) is placed on the exposed surface. After a few seconds a drop of a saturated aqueous solution of magenta is added. The excess stain is drawn off with blotting paper. Destaining to a desired density is accomplished by carefully passing absolute alcohol over the style absorbing it at the basal end with filter paper, old linen cloth or blotting paper. Several drops of glycerin and a cover glass complete the mount.

If the pollen-tubes are found to be near the base of the style the ovary may be sectioned free-hand by using a razor and a piece of pith. Each section is then placed on a slide and stained as described for the style. Pollen-tubes can thus be traced into the micropyle.

The time required for staining and the amount of destaining varies with styles from different species of plants. This stain gives a dark red color to the cytoplasm of the pollen-tubes while the surrounding tissue is slightly stained. Altho mounts of this type are not fully permanent they keep their color for six months or a year after they are made.

[1]The dyes used in developing this technic were obtained from Eimer and Amend, and were labeled as follows: "Carmine No. 40." "Aniline red A12 (magenta)," and "Aniline blue (water soluble)."

This method is also applicable to pistils whose styles are solid. If the style is short and thick, free-hand sections may be made and placed on the slide. In some cases (*Brassica* and *Convallaria*) the entire pistil may be thus sectioned and stained. If tissue differentiation is difficult a drop of anilin blue may be added just before the magenta. The cytoplasm of the pollen-tubes will stain blue and the surrounding tissue will stain red.

This method of staining pollen-tubes has many advantages. The material may be fixed and stored for study at one's convenience. This method has been found effective for every species studied except *Feijoa sellowiana* which has a hard, thick, style that is red in color. The method is simple and certain. Prepared slides may be kept for later study and comparisons. The stain is a cytoplasmic stain and not a cellulose and callose stain.

The excellent results obtained in the case of both hollow pistils and solid styles seem to indicate that differential staining of pollen-tubes in many plants might be simplified in this manner.

LACMOID-MARTIUS-YELLOW FOR STAINING POLLEN-TUBES IN THE STYLE

B. R. NEBEL, *N. Y. Agricultural Experiment Station, Geneva, N. Y.*

Lacmoid or Rescorcin Blue (C. I. No. 908) was first employed by Tsvett (see Conn[1]) as a specific callose stain. It is also mentioned by Strasburger[2] and Krause,[3] has been described by Cooper [4] used on fresh material, and by Bradbury[5] for both fresh and sectioned material. Cooper prescribes "to boil fresh material (pollinated styles), for two minutes in N/10 acetic acid after which the outer integument of the pistil with its accompanying hairs could be removed without injury to the internal tissues. Staining was accomplished by using an aqueous solution of lacmoid after bringing the material to a neutral or slightly alkaline reaction."

For purposes of greater contrast, useful for visual observation as well as for photomicrographic records, the present author uses the following procedure.

Material is collected in small vials in which it is kept from drying out by the addition of a drop of water. Slender styles and ovaries are crushed between two slides. Larger ones are sectioned longitudinally by hand, and the sections then squeezed as above to permit penetration of the aqueous stain. The material is either stained on the slide or immersed in the stain in pieces in a small dish. The stain: 5 mg. of lacmoid and 5 mg. of martius yellow (C. I. No. 9) are dissolved in 10 to 15 cc. of water. Both dyes used in the present work were National Aniline Co. products; including two lots of martius yellow, both certified stains, (Cert. Nos. NMy1 and NMy2), having dye contents of 59 and 79% respectively. The solution should show a deep olive green color which is easily attained by adding a few drops of a 1% solution of ammonia, bringing the solution to pH 8.

After two to five minutes of staining the material is mounted in the stain or else in water of the same pH. With the use of a somewhat powerful light, no difficulty was encountered from the opaqueness of

[1]Conn, H. J. Biological Stains. Geneva, N. Y. 1929. See p. 74.

[2]Strasburger E. Das botanische Praktikum. Gustav Fischer, Jena., 1921. See p. 805.

[3]Krause, R. Enzyklopaedie der Mikroskopischen Technik. Urban and Schwarzenberg, Berlin 1926–7. See Vol. 3, p. 2021.

[4]Cooper, J. R. The behavior of pollen tubes in self and cross pollination. Proceedings of the American Society for Horticultural Science, **25**, 138–140. 1928.

[5]Bradbury, Dorothy. A comparative study of the developing and aborting fruits of *Prunus cerasus*. Am. J. Bot., **16**, 525–542. 1929.

uncleared tissues. A photomicrograph showing the penetration of tubes in a self-pollination of the Bartlett pear is given. (Fig.1)

To make permanent mounts, different procedures were followed. Other modifications of the same process may undoubtedly be found useful for different plant material: 1) Crush, dissect or otherwise prepare fresh material. 2) Fix material in Carnoy's solution. 3) Transfer via 70% alcohol to alkaline water. 4)Wash thoroly in tap water to remove all acid or excessive alkali. 5) Transfer to stain for

FIG. 1

FIG. 2

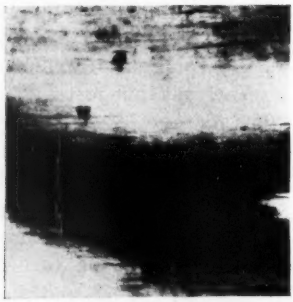


Fig. 1. Style of Bartlett pear 24 hours after self pollination. Fresh mount in water. Stained with martius yellow and lacmoid. Tube 160 mm. Obj. 21x, Oc 10x Camera Leitz Makam. Wratten M. Plate without filter. Incandescent projection bulb.

Fig. 2. Style of ornamental gladiolus open pollinated mounted in balsam. Stained with martius yellow and lacmoid. The pollen tubes being relatively old, only the callose plugs show. Tube 160 mm. Obj. 10xOc 15x Camera Leitz Makam. Wratten M plate. Wratten E Filter 22 orange. Light as above.

1 to 5 minutes. 6) Dehydrate rapidly in alcohols containing lacmoid in solution in its blue modification (pH 8). 7) Mount in balsam via xylol or in cedar oil. The latter mount should be sealed. In certain cases it was found useful to take step 1 after 2 or 3. The most successful concentration of the stain, duration of its action as well as the following steps have to be varied according to the material in question. Diaphane was found unfit as a mounting medium on account of the stain fading out. The same was true of glycerin jelly.

Fig. 2 is a photomicrograph from a permanent mount in balsam, of an open pollinated ornamental gladiolus. The slide was exposed to daylight for three weeks and then photographed. Only the callose plugs of the tubes are visible as big black dots. (See also explanation of figures.)

The method is fast and easy to apply. It is believed that the callose stain permits of interpreting the physiologic condition of the tube more readily than a stain of the plasmatic content alone. The method proved to be particularly valuable with fleshy styles such as are found in pomaceous plants, cherries, plums, and grapes. It was also exercised on ornamental gladiolus, snapdragon, mint, and evening primrose.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order direct from STAIN TECHNOLOGY, at a price of 6 cents each.

BUJARD, EUG. Une coloration strictement élective des granulations basophiles des mastocytes. Bull. d'Histol. Appl., **7** 264–269. 1930.

This is a method for the elective staining of basophilic granules in mastocytes. Fix in either alcohol, formalin-saline, alcohol-formalin, bichromate-formol. Stain deparaffined sections in 1% aqueous acid fuchsin, 30 seconds. Rinse rapidly in water. Treat with a 0.8% aqueous solution of bromine, 4 to 5 minutes, until violet, in a closed container (the exact concentration is very important). Wash in water. Differentiate in 95% alcohol or acid alcohol (1% HCl), 1 to 3 minutes, until light mauve. The fuchsin precipitate should be dissolved out of all the preparation except the granules. Complete differentiation and dehydration with absolute alcohol, exercising care not to destain mastocytes. Clear in xylol; mount in balsam. The granules are carmin red, while the nuclei and cytoplasm are colorless.

Nuclei may be stained after the granules if desired. In this case rinse in water after differentiation of the granules. Mordant in 4% iron-alum 4 minutes at 50°C or 40 to 50 minutes at room temperature. Stain with Regaud's hematoxylin for the same time at the same temperature. Differentiate in 1% iron alum and proceed as usual, timing carefully the alcohols. Nuclei are black, granules red. Tongues of white rats have served for material. Slides have not faded for a period of two years.

Various acid and basic stains were tested for the reaction. Of the acid stains all except fuchsin gave negative results. The reaction is positive with the following basic stains: fuchsin, methyl green, and violet. Basic fuchsin and Merck's "diamant fuchsin" proved as good as acid fuchsin, but granules are of a rather purplish red. Thionin, toluidin and methylene blue are also good, the reaction is dark blue, slightly metachromatic.

In order to understand the nature of the reaction, bromine was replaced by various acids or oxidizing agents using the same three stains: acid fuchsin, basic fuchsin and toluidin blue. Toluidin blue alone gave positive results with acids. Basic fuchsin and toluidin blue with oxidants and Lugol's solution. Bromine alone succeeds with acid fuchsin. The reaction with the basic stains is not similar to that of acid fuchsin for the first are direct stains, and the second is a secondary stain. (The mastocytes are colorless before the action of bromine).

The author thinks that the reaction may be due to an energetic adsorption of the precipitate by the basophilic granules, but it is more probably an elective affinity of the granules to the new stains formed during the reaction.

CAPINPIN, J. M. Brazilin stain on smear preparations of Oenothera Pollen Mother Cells. Science, **72**, 370–371. 1930.

The following procedure based mainly on Belling's original method is especially recommended for Oenothera.

Clip off segments of anthers and arrange side by side on a slide. With a second slide held crosswise squeeze circularly with strength enough to extrude the mother cells. Invert both slides immediately over parallel supports placed in a dish containing the fixative for 2 to 6 hours. This fluid consists of 2 solutions mixed 1:1 just before application: A) chromic acid, 5 gr.; glacial acetic, 50 cc.; water, 320 cc.; B) formalin, 200 cc.; water, 175 cc., (or, especially for metaphases, formalin, 100 cc.; water, 275 cc.)

Transfer slides, right side up, to 4 parts of water and 1 part of the solution A for 10 to 15 minutes and remove fragments and walls. Run thru 15%, 30% and 50% alcohol,

and leave over night in 70%. Mordant in a freshly prepared solution of 1% iron alum in 70% alcohol for at least 12 hours. Wash 30 to 60 minutes in 70% alcohol. Stain in 0.5% solution of brazilin[1] in 70% alcohol, ripened a week, for 2 to 6 hours. Wash in 70% alcohol and differentiate in 1% solution of iron alum in 70% alcohol. Chromosomes should be brownish black or black, the cytoplasm pink or straw colored and the cell walls colorless. Wash in 70% and 95% alcohol. Pass thru absolute alcohol, 1 part of absolute alcohol to 1 part of cedar oil, 1 part of xylol to 1 part of cedar oil, 9 parts of xylol to 1 part of cedar oil, and finally thru pure xylol. Mount in Canada balsam.

Sharp and well defined figures of all stages of the chromosomal cycle are thus obtained.

HARRIS, MINNIE B. K. A simple method for staining spirochaetes. Science, **72**, 275. 1930.

This is a modification of the method of Kliewe (Centbl. f. Bakt., I Abt., Refr., **76**, 232 1924). A film is prepared as usual, air-dried and fixed by passing several times thru a Bunsen flame. It is mordanted 8 to 10 minutes with a 1% aqu. sol. of potassium permanganate, washed in water, stained 8 to 10 minutes with a 2% aqu. sol. of methyl violet (exact nature of dye, or source not mentioned), and finally washed in water. In staining *Treponema pallidum* it is often desirable to warm the mordant gently on the slide; but it is never necessary to warm the stain. The shorter staining periods are for the coarser spirochaetes, the longer periods for the more delicate varieties. With this method the spirochaetes are stained bluish black, and stand out very clearly.

LEIFSON, EINAR. A method of staining bacterial flagella and capsules together with a study of the origin of flagella. J. Bact., **20**, 203–211. 1930.

Growth from the base of an agar slant, or from a bouillon culture recentrifuged and resuspended in distilled water, is smeared over a scrupulously clean slide.

The following stain is recommended: Ammonium or potassium alum, sat. aqu. sol., 20 cc.; tannic acid, 20% aqu. sol. 10 cc.; dist. water, 10 cc.; ethyl alcohol, 95%, 15 cc.; basic fuchsin, sat. alc. sol., 3 cc. Crystal violet may be used in place of basic fuchsin by employing 1.5 cc. instead of 3 cc. of the saturated alcoholic solution; or methylene blue by using 5 cc. and cutting down the additional 15 cc. of ethyl alcohol to 10 cc. The ingredients in this stain are to be mixed in order given; the solution remains good a week or more.

The author does not state dye content, chemical nature or source of dyes employed. This might make it difficult to duplicate the staining formula, particularly in the case of basic fuchsin. Some fuchsins are chlorides, and some acetates; and as these two compounds differ much in solubility in alcohol, the above staining formula might vary considerably in strength according to which fuchsin one employed.

Stain for 10 minutes in a warm room, or in an incubator if the temperature of the room is low. After washing the preparation is satisfactory so far as concerns the flagella themselves. A counterstain may be used if it is desired to color the organisms a different hue from their flagella. A good counterstain when fuchsin has been employed is 0.1% aqueous methylene blue with 1% borax; or if the flagella are blue a good counterstain is carbol fuchsin diluted 1/10.

ZIKES. Die Geisselfärbung. Zentbl. f. Bakt., II Abt., **81**, 161–166. 1930.

The writer gives a summary of several previously described methods of staining bacterial flagella, describing the results obtained with them. Also proposes the following modification of the Loeffler mordant, which is superior to the latter in that it can be kept a long time without precipitation.

On a water bath dissolve 2 g. tannin in 8 cc. water, and allow to cool. To this add drop by drop 5 cc. of a saturated cold solution of chrom alum; then 1.5 cc. of 1% osmic acid, and finally 1 cc. of a saturated gentian violet solution (not stated whether aqueous or alcoholic). This mordant is used cold and staining follows with freshly prepared anilin gentian violet, Ehrlich. (No information is furnished as to the source from which this dye or any other employed in the writer's work was obtained.)

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[1]Thanks to a personal letter from the author we learn that Grübler brazilin was employed, purchased from the Arthur H. Thomas Co. of Philadelphia.